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THE EFFECTS OF ACUTE GAMMA IRRADIATION ON THE BRINE SHRIMP, *ARTEMIA*. II. FEMALE REPRODUCTIVE PERFORMANCE[1, 2, 3]

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The effects of acute gamma irradiation on adult life span and male reproductive performance were reported in the first paper of this series (Squire and Grosch, 1970), along with the Materials and Methods, and the general rationale behind the experiments. The present paper records the effects on female reproductive performance.

The earlier reports of Grosch and Erdman (1955) and Grosch and Sullivan (1955) described some of the criteria of x-ray damage to female reproductive performance. However, these data were dependent upon culture techniques which have since been modified. In addition, these reports did not provide an analysis according to the gametogenic stage irradiated. The report of Grosch (1962) was restricted to the analysis of populations of irradiated ancestry, and did not concern the treated generation itself.

Cervini and Giavelli (1965), Giavelli (1966), Giavelli and Cervini (1966), and Metalli and Ballardin (1962) restricted their studies to fecundity and fertility analyses of viviparous broods after oocyte treatment of various parthenogenetic species of *Artemia*.

Results

Fecundity

The statistical procedures were the same as those described for the life span studies (Squire and Grosch, 1970). Fecundity was defined as the sum total of viviparous (nauplii) and oviparous (cysts) gametes produced by a given female throughout her life. All treatments significantly reduced fecundity (Table I). A dose of 5 kR resulted in greatly reduced fecundity ($\bar{X} = 43$), while doses of 10 50, and 100 kR resulted in total infecundity. Since the mean life span of the 5 kR

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TABLE I

Average fecundity and number of broods per irradiated female, with one-tailed t test comparisons of treated versus *control animals*

Level	Dose (kR)	Fecundity/female		No. of broods/female	
		$\bar{X} \mp$ S.E.	t	$\bar{X} \mp$ S.E.	t
1	0	1695 ∓ 329	—	10.1 ∓ 1.8	—
3	1	919 ∓ 216	1.974*	7.0 ∓ 1.3	1.569
4	2	289 ∓ 134	3.961**	4.2 ∓ 1.1	3.123**
5	5	43 ∓ 17	5.021**	1.1 ∓ 0.3	5.800***

* Significant at 0.05.
** Significant at 0.01.
*** Significant at 0.001.

females was virtually identical to that of the controls, this effect on fecundity is independent of life span.

The mean differences between replicates within treatment levels were not significant for any dose at 0.05. The F test for variances between replicates within treatment levels was significant for level 5 (5 kR). This difference was due to a single female which produced 1255 gametes, when the group average excluding her contribution was 66. She was 17 standard deviations from the mean, and none of her cysts hatched after the first brood. Accordingly the data from this female were not used in the fecundity analysis. Adjusted means, variances and degrees of freedom were used for comparisons involving this treatment level.

Fertility

A similar analysis was conducted for fertility (Table II), which was defined as the sum total of the nauplii produced as viviparous broods plus those which hatched from oviparous cysts. The difference in variances (but not means) was once again significant for 5 kR. The separate means were 4.6 and 0.2. Appro-

TABLE II

Average fertility per female and per cent hatchability per brood, with one-tailed t test comparisons of treated versus *control animals*

Level	Dose (kR)	Fertility/female		% hatchability/brood	
		$\bar{X} \mp$ S.E.	t	$\bar{X}$	t
1	0	1318 ∓ 358	—	18.5	—
3	1	718 ∓ 201	1.46 (0.10)	21.0	(0.503)
4	2	64 ∓ 30	3.49**	12.1	1.824*
5	5	2.4 ∓ 1.7	3.68**	2.2	7.412*

* Significant at 0.05.
** Significant at 0.01.

priate pooled values were nevertheless obtained for all treatment levels and comparisons made (Table II). The lack of significance at 1 kR may be ascribed to the presence of a few females which produced large numbers of cysts with low hatchability. This comparison is significant at the 0.10 level.

As may be seen in Tables I–II, the average fecundity and fertility per female decreases with increasing dose. The same trend is found when the per female values are computed for average brood size, largest brood size per treatment level, the average of the largest single brood for each of the fecund females, or the average largest brood of the three best females (Table III).

Cyst hatchability

Hatchability tests for all oviparous broods were conducted and analyzed in the same manner as previously described for males. Untransformed treatment

TABLE III

Evidence for a reduction in the number of competent oogonia as a result of gamma irradiation of adult female brine shrimp

Level	Gamma Ray Dose (kR)	Avg. size of brood		Largest single brood value in treatment level		Avg. of the largest single brood produced by each female		Avg. fecundity of the largest single brood produced by each of the 3 most productive females
		Fec.	Fert.	Fec.	Fert.	Fec.	Fert.	
1	0	168	134	485	485	268	240	423
3	1	130	102	374	374	191	179	310
4	2	69	15	217	99	97	26	177
5*	5	57	1	238	18	71	3	133

* The values for level 5 include the exceptionally fecund female described in the text.

means and t test comparisons are given in Table II. While 1 kR did not decrease hatchability, 2 kR and 5 kR did so. The differences of 1 kR *vs.* 2 kR, and 2 kR *vs.* 5 kR are also significant.

Number of broods

The average number of broods per female was calculated in the same manner as the fecundity data. The reduction in brood number was highly significant at doses of 2 kR and more (Table I). The average interval between broods was calculated per female (Table IV) and found to increase markedly at 2 kR and 5 kR. The average percentage of gametes produced as cysts per female also rises at these levels (Table IV).

Survival to adulthood and sex ratio

The percentage of the nauplii (viviparous or hatched from cysts) to reach maturity was also calculated for broods I and IV. Survival to adulthood measures late dominant lethal events, since, as measured here, it excluded all pre-

TABLE IV

Average brood intervals, percentage of oviparous gametes, and the fraction of encysted broods which failed to hatch after adult females were irradiated

Level	Gamma Ray Dose (kR)	Avg brood interval (days)	Avg % of gametes deposited as cysts female	Fraction of encysted broods which failed to hatch
1	0	3.74	41.3	1/32
3	1	3.53	38.5	3/21
4	2	6.89	86.4	1/23
5	5	9.08	85.7	14/17*

* Nine-tenths of these broods were produced by one exceptional female; see text.

naupliar deaths from analysis. Analysis of brood I was restricted to those broods which were deposited between the fourth and sixth days after treatment, and thus were derived from irradiated oocytes or oocyte-nurse cell complexes. If the time sequence published for the diploid parthenogenetic species from Sète (Cervine and Giavelli, 1965) is applicable to this species, then most of these oocytes were in prophase I at time of treatment, and some may have reached metaphase I. None of these gametes were post metaphase I at time of treatment, since in order to be fertilized the eggs must be in the oviducts when copulation occurs; and such eggs are in metaphase I at that time.

Brood I was analyzed as 2×4 contingency tables (Steel and Torrie, 1960) for survival to adulthood and sex ratio. Survival to adulthood was significantly reduced with increasing dose ($P < 0.005$). There was no significant change in sex ratio (Table V).

Brood IV animals were derived from treated oogonia. The survival and sex ratio data are summarized in Table V. None of these values are significant, although the survival value for level 4 might have reached significance with larger

TABLE V

The survival to adulthood and sex ratio of offspring from ten irradiated females per gamma ray dose level shown

Brood #	Level	Dose (kR)	No. of nauplii	No. of survivors	% survival	Sex ratio % males
I[a]	1	0	157	129	82.2	50.4
	3	1	53	36	67.9	52.8
	4	2	50	21	42.0	52.4
	5	5	18	6	33.0	33.3
X^2					40.25**	0.95NS
IV	1	0	1147	582	50.7	48.7
	3	1	581	252	43.4	49.6
	4	2	55	20	36.4	60.0
	5	5	—	—	—	—
X^2					1.04NS	0.18NS

[a] Brood I data selected for days 4–6 only; see text.

** Significant at 0.01.

samples. Survival was also analyzed by using t test comparisons of percentage data following the arcsin $\sqrt{\%}$ transformation, as well as with the contingency table shown.

Additional observations

Gross observation of females of replicate 1 on the 22nd day after treatment revealed full ovisacs in all cases after 5 and 10 kR. Nevertheless, two of the five females given 5 kR and all of the three surviving 10 kR failed to produce recoverable nauplii or cysts subsequent to this observation. No excretory gland abnormalities were noted.

Cysts from irradiated females were often orange and translucent, while normal cysts are opaque and brown. Cysts deposited after 5 kR exposures were particularly abnormal in this respect, and were also characterized by frequent brittleness (which resulted in cyst breakage when touched with the dissecting needle during hatchability studies) and flattened cysts approaching a disc shape. Since all cysts were treated and stored under identical conditions, the abnormality is attributed to experimental treatment, rather than to attendant conditions of the experiment. Broods in which translucent cysts predominated showed poor hatchability and particles resembling cysts occasionally degenerated completely during a brief storage period on filter paper.

Some pair matings were set up from first-brood individuals. Offspring from the 5 kR series failed to reproduce (two full-sib matings plus three females crossed to normal males). Eight full-sib matings from the 2 kR series produced cysts but no nauplii. The offspring from 1 kR females produced both nauplii and cysts. Mortality was high in the 2 kR and 5 kR offspring following sexual maturity. The cysts produced by these X_1 individuals were not tested for hatchability in most cases, and full life history data were not obtained.

A few morphological abnormalities were scored in the X_1 of treated females. Unfortunately, these animals usually failed to reproduce. The fusion of adjacent appendages to each other, or to genitalia, was the most common trait. Abnormally small eyes or claspers were asymmetrical traits, and abnormal reproductive organs (only one functional ovary), bent tails, missing eyes or claspers were also noted.

Discussion

Factors affecting female reproductive performance

Female fecundity was defined as the sum total of all recoverable gametes produced by a single female throughout her reproductive history. Such a measure is the final result of many interacting factors. Presumably a cohort of oogonia is produced from a smaller number of stem cells. On an average of every three and one-half days, these oogonia then produce a ribbon of cells which in time differentiate into an oocyte and a nurse-cell complex. Nurse cells become polyploid and contribute to vitellogenesis. The blood cells have also been suggested to play a role by transporting materials to the ovarian region. Finally, a differentiated oocyte is produced which enters the median ovisac (uterus) and is quickly fertilized. (For a more comprehensive coverage, see papers by Anteunis, Fautrez-Firlefyn and

Fautrez, 1966a, 1966b; Bowen, 1962; Cassidy, 1965; Fautrez-Firlefyn, 1951; Lochhead, 1950; Lochhead and Lochhead, 1941, 1967.)

In the author's opinion, *Artemia* oogonia comprise a nonexhausting stem-cell population in the adult female; and a single oogonium may contribute to each successive brood. According to this view, the stem-cell population would be comprised of "primary oogonia." Every three and one-half days, each primary oogonium would divide, producing one primary oogonium and one "secondary oogonium." This secondary oogonium would then undergo a series of mitotic divisions and produce an oocyte-nurse cell complex.

Normally, the second cleavage division occurs about five hours after the descent of the eggs into the uterus, and shell formation is initiated at this time in oviparous broods (Fautrez-Firlefyn and Van Dyck, 1961). Unfertilized eggs may also be encysted (Squire, unpublished data). In the case of viviparous broods, the zygotes must differentiate into a swimming nauplius in order to be scored. In the case of oviparous broods, any gamete which passes into the uterus will probably be recovered so long as shell deposition is approximately normal. Cyst hatchability, then, is a measure of dominant lethal events. Factors which may conceivably affect cyst hatchability include (1) failure of fertilization, (2) nutritional inadequacies of the oocyte resulting from damage to the oocyte, to the nutritive cells of the complex, or to general physiological disturbances in the females, (3) improper shell deposition, and (4) genetic lethality. Reabsorption, either of gametes prior to fertilization, or of viviparous zygotes after fertilization, might also be a factor in scoring fertility and fecundity.

Fecundity was defined as the total number of recoverable gametes. Thus, it would include (1) any change in the number of oogonia actually present at the time, (2) induced changes in the inherent capacity of those oogonia which were present to produce functional gametes, (3) any nutritional changes which led to the failure of a differentiating oocyte to be recovered (such as the absence of vitellogenesis or reabsorption of the oocyte), (4) reabsorption of viviparous zygotes and (5) failure to score partially developed viviparous zygotes, which had been expelled, prior to their disintegration.

If the concept of an oogonial stem-cell component is correct, then any change in the general health of a female *Artemia* may temporarily or permanently alter the number of gametes recovered per brood. Such a decrease could occur without altering the actual number of oogonia present in the ovaries. Although we have no information concerning the effects of irradiation on the physiology of *Artemia*, presumably such effects exist, and quite possibly nutrient utilization is altered.

Some information does exist concerning various aspects of brine shrimp nutrition. Lochhead and Lochhead (personal communication) reported that oocytes may fail to differentiate in starved animals. They believe that oogonial number is more strongly influenced by nutrition than by age. Subsequent events in our laboratory would tend to support some of their conclusions. Greatly reduced fecundity and stage-specific patterns of larval mortality appeared to be associated with a lack of algae in the diet.

D'Agostino and Provasoli (1968) demonstrated that the inter-relationship of algal diet, salt concentration, other nutritional requirements, and fertility is not

a simple one. Reduced salinity resulted in depressed fertility unless specific nutrients were added to their synthetic media.

Fertility, defined as the sum total of the recovered nauplii from viviparous and oviparous broods, combines the factors included in fecundity and hatchability. It should be pointed out that we still do not know what factors control the mechanism of cyst deposition. The frequency of cyst production is generally felt to increase as a response to stress, but normal females also produce oviparous as well as viviparous broods. Various environmental factors such as diet, temperature and salinity have been implicated at one time or another.

Fecundity of irradiated females

Female fecundity was progressively reduced at doses of from 1 kR to 5 kR. No recoverable gametes were found at 10 kR or higher doses. The data summarized in Tables I–III indicate that the total number of oogonia which successfully contribute to each brood has been progressively reduced with increasing doses. However, no cytological study of the number of oogonia actually present in these females has been made.

The largest single brood per female, the single largest brood per treatment, and the average of the three largest brood values (on a per-female basis) per treatment group all progressively decrease with increasing dose. This leads me to hypothesize that gonial cell lethality occurs at all doses of 1 kR and above, although the other factors discussed above should not be ruled out.

Typically, the first brood produced by a female *Artemia* is relatively small, but subsequent broods are larger. This suggests that the young female may have fewer competent oogonia capable of contributing to each brood, but the number of such oogonia increases rapidly following sexual maturity. Whether this increase is due to recruitment from already existing "dormant" oogonia or to multiplication of stem-cells cannot be determined at the present time. In either case, a differential radio-sensitivity is likely to occur, with actively dividing cells being the most sensitive to damage. Such damage would be expected to give the kind of results reported here.

Although two reports (Grosch and Erdman, 1955; Grosch and Sullivan, 1955) indicated the sterilizing dose for premeiotic and early postmeiotic stages, there have been no reports directly concerned with gonial cell sensitivity in *Artemia*. The silkworm, *Bombyx*, is perhaps the closest biological system with which we can currently make comparisons. This Lepidopteran has 28 pairs of minute and presumably holokinetic chromosomes, as compared to 21 pairs of holokinetic chromosomes in the common Californian race of *Artemia*. Tazima and Kondo (1963, page 246) concluded that the LD-50 is about 1000 and 2000 R for spermatogonia and oogonia, respectively, when exposed to Cs-137 acute gamma irradiation. Ballardin and Metalli (1968) state that an oogonial dose of 1 kR had no effect on the fecundity of the diploid parthenogenetic species from Sète, when measured over a 20-day period. Their abstract gave no data concerning this point.

Cyst production and hatchability

The hatchability data show no detectable dominant lethality at 1 kR, although such events do occur at 2 kR and higher doses. The high frequencies of abnormal

cysts recovered from 5 kR females suggest that the shell glands are damaged by this dose, and lower levels may also have been affected. Such abnormal shell deposition could easily alter the resistance of the embryo to normal environmental conditions such as drying, and thus by reflected in hatchability and fertility data. Since cysts from unfertilized females appear normal, it is concluded that genetic dominant lethals which were induced in the oogonia would not affect cyst morphology.

The analysis of hatchability data excluded all encysted broods with zero hatchability. This exclusion does not seriously affect the data as summarized in Table II for the following reasons. Such broods are extremely rare at all levels except 5 kR (Table IV). At this level the average hatchability already approaches zero ($\bar{X} = 2.2\%$). The inclusion of broods with zero hatchability would reduce this value still further, while values for the other levels would remain substantially the same as presented. Excluding such broods is an attempt to separate other dominant lethal events from the failure of fertilization. The latter condition will produce broods with zero hatchability.

Repeated failure of copulation often results in the accumulation of several unexpelled egg clutches within the female. Gamete degeneration becomes evident, with pronounced accumulations of yolky material and darkening of the excretory glands (Lochhead and Lochhead, 1941) which may result in temporary or permanent infecundity (M. Lochhead, personal communication). Some females successfully expell numerous egg clutches in the absence of copulation and thus escape this syndrome. Expelled material is usually in the form of cysts, but undeveloped products lacking cyst walls have been recovered. Expulsion of partially developed embryos has also been observed in females after a fractionated dose of 5 kR (1 kR of gamma radiation per day for five days; Squire, unpublished data). These embryos degenerate rapidly at summer temperatures and are easily overlooked. On the other hand, on several filter papers used to separate encysted broods I failed to find cysts after a storage period and suspect that the objects filtered may have been undeveloped eggs without cyst walls.

The average per cent of gametes deposited per female (Table IV) must be viewed with these factors in mind. Nevertheless the difference in values at 2 kR and 5 kR is outstanding. This difference becomes even more remarkable upon observing that at 5 kR all recoverable broods consisted solely of cysts when premeiotic stages were irradiated, and the same was usually true for the 2 kR series as well. Viviparious broods were frequent after 1 kR and in the controls.

Brood interval and number of broods

The average interval between broods also increased in the 2 kR and 5 kR levels (Table IV). Examination of individual female records suggest this increase was due to the failure of whole broods to be recovered, rather than to a simple lengthening of the interval between broods. This observation cannot be explained simply by complete dominant lethality in the 2 kR series, since most encysted broods showed some hatch; and a number of viviparous broods were scored. Complete dominant lethality could account for the absence of viviparous broods in the 5 kR series, however, since cyst hatchability approached zero for this group. Failure of fertilization may also explain the 5 kR results, but once again the

relative infrequency of encysted broods with zero hatchability makes this explanation difficult to accept for the 2 kR series (Table IV). The observation of Grosch and Erdman (1955, page 280) bears repeating: "the neighborhood of 2000 R is critical not only for numbers of broods produced but on the basis of inhibition of viviparity."

In earlier research, an acute dose of 2500 R x-days resulted in a cessation of female gamete production, while 2250 R or less did not prevent continued reproduction by cells which were premeiotic at the time of treatment (Grosch and Sullivan, 1955). Acute x-ray doses of 4080 R or more, usually resulted in females with empty uteri, although exceptional females produced a single brood from cells which were prezygotic at the time of irradiation (Grosch and Erdman, 1955). In the present experiment, full uteri were observed at 5 and 10 kR, although no gametes were recovered from 10 kR females and 5 kR females were highly infertile. The earlier and recent experiments differed in several factors. In 1955 sea water rather than brine was used. This resulted in a shortened life span for irradiated and control animals, and probably imposed an additional stress as well. The reproductive behavior of treated specimens of *Artemia* would thus reflect the combined effects of stress due to irradiation and suboptimal salinity. Squire (unpublished data) found that the fertility of stock #3 animals cultured in plain Instant Ocean was only 14% that of animals maintained in the improved medium, while Grosch (1962) found that the adaptive values of experimental cultures varied with salinity.

In the present experiment, several 5 kR females were characterized by extremely late dates of brood I deposition. A detailed analysis shows that of the 10 females treated, one produced 11 broods, four produced two broods each, two produced one brood each, and two failed to produce any recoverable broods. In all but one case, these broods were probably oogonia at the time of treatment, suggesting that 5 kR resulted in complete dominant lethality of oocytes irradiated prior to metaphase I.

When the life span data of Grosch and Erdman (1955) are superimposed on the present reproductive histories of various treatment levels, the number of broods per female is quite similar in the two experiments. We may therefore conclude that most, if not all, of the discrepancies between the two experiments stem from differences in life span. As they point out, the first brood deposited in their experiment usually represented cells which were postmeiotic at the time of treatment.

X_1 mortality, sex ratio, and reproductive performance

Brood I data for survival to adulthood, adult mortality and reproductive patterns demonstrate the presence of genetic damage in those animals descended from 2 kR and 5 kR females. Genetic damage at a lower dose has been reported for diploid parthenogenetic *Artemia* from Cagliari. A reduction in the fertility of irradiated and X_1 females followed a 1 kR dose of x-rays to prophase oocytes (Metalli and Ballardin, 1962). This reduction was significantly greater in the X_1 than in the treated generation. They obtained similar results with the tetraploid parthenogenetic species from Comacchio, except that the effect was

less pronounced, and there was no significant difference between the X_1 and treated generations.

Survival to adulthood of nauplii or hatch derived from irradiated oogonia (brood IV) demonstrated a decrease in average values with increasing dose, but was not significant. Ballardin and Metalli (1968) state that an oogonial dose of 1 kR had no effect on the survival to adulthood of X_1 diploid parthenogenetic *Artemia* from Sète.

As would be expected, the results of various radiation experiments differ according to the polyploid level and the type of meiosis (regular or variously modified) characteristic of the particular *Artemia* species. Additional data from these species will provide a unique opportunity for comparative studies.

The failure of any treatment to seriously affect the sex ratio suggests that the differential segment of the Y-chromosome may not contain many viability loci in the heterogametic female.

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Summary and Conclusions

Treated females were completely infecund after doses of 10–100 kR. Reduced fecundity resulted from doses of 1–5 kR. This is ascribed to oogonial lethality, and it is proposed that the *Artemia* ovary contains a non-exhausted oogonial stem-cell component which contributes to each successive brood. Additional causes of reduced brood size may be nutritional inadequacies and other physiological damage induced by the treatment. Sterility was almost complete after 5 kR.

Cyst hatchability data revealed no detectable dominant lethality after 1 kR, although such effects did occur after 2 and 5 kR. Some of the failure in hatching reflects probable physiological damage to the shell glands, as well as other physiological and genetic components.

The average number of broods was significantly decreased after 2 and 5 kR. Much of this reduction resulted from the absence of entire broods from the recorded data. Viviparity was also inhibited at these doses.

Data for survival to adulthood, adult mortality and reproductive patterns demonstrate that genetic damage was present in those animals descended from oocytes which had been treated with 2 or 5 kR. No definite decrease in survival to adulthood was found in animals descended from treated oogonia after 1 or 2 kR. Sex ratios were not significantly changed with any dose.

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